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Characterization of Carotene Accumulation in *Ustilago violacea* Using High-Performance Liquid Chromatography

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Abstract. Quantitative analysis of carotene accumulation in white, pink, pumpkin, orange, and yellow haploid strains of *Ustilago violacea* by high-performance liquid chromatography indicated that specific patterns of carotene accumulation are primarily responsible for the white, pumpkin, orange, and yellow phenotypes. The yellow strains accumulated primarily β-zeacarotene and β-carotene. The white strains accumulated primarily the colorless carotene, phytoene, or did not accumulate any carotene at all. Carotene accumulation in pink haploid strains followed the same patterns as for the white, pumpkin, orange, or yellow strains. Pink diploid and disomic strains of *U. violacea* with various parental combinations of the color mutations accumulated either cis-β-zeacarotene and β-carotene or only β-carotene. The pattern of carotene accumulation in conjunction with the available genetic information for the carotene loci in *U. violacea* was used as a basis for the construction of a new genetic model for carotene biosynthesis in *U. violacea*. The model employs three dehydrogenases and one cyclase for the synthesis of β-carotene from phytoene, and accounts for the carotene accumulation patterns of either cis-β-zeacarotene and β-carotene or lycopene, γ-carotene, and β-carotene.

The phytopathogenic basidiomycete *Ustilago violacea*, a bipolar (a_1, a_2) heterothallic species that causes anther smut in *Silene alba*, accumulates carotenes. Extensive color polymorphism was demonstrated in a collection of wild strains of *U. violacea* obtained from smutted anthers in the field or from herbarium specimens [15]. White (*w*), yellow (*y*), pumpkin (*p*), and pink strains were recovered in this survey [15]. Orange (*o*) and *y* color mutants of *U. violacea* were obtained after ultraviolet mutagenesis of pink sporidia [8, 9]. White mutants were isolated from pink, *o*, and *y* strains by ultraviolet mutagenesis [14].

Genetic analysis of the color loci indicated that *o*, *p*, *y*, and *w* are tightly linked (~1 cM) with the white locus in one chromosome arm and the remaining loci in the other chromosome arm [3, 14, 16]. Further analysis of the white locus indicated at least two complementation regions [12].

Biochemical analysis of the pigmented strains of *U. violacea* revealed that yellow sporidia accumulated β-carotene, orange sporidia γ-carotene, and pumpkin lycopene [14, 16]. While pink strains accumulated cytochrome c [33], ultraviolet-induced white strains of *U. violacea* had no detectable carotene, and intermediates of carotene biosynthesis were generally not detected. A Porter-Lincoln pathway for carotene biosynthesis was proposed for *U. violacea* [14].

In this study, we examined carotene accumulation in *U. violacea* qualitatively and quantitatively by screening representative groups of haploid white and pink strains, as well as the pumpkin, orange, and yellow strains using high-performance liquid chromatography (HPLC). In addition, diploid and disomic strains of *U. violacea* with various parental combinations of the color mutations (*w*, *p*, *o*, and *y*) were also characterized with respect to carotene content. Our results and the genetics of the color mutations provided the information for a new model of genetic control in carotenogenesis in *U. violacea*.

Materials and Methods

Strains of *U. violacea* were grown for seven days at 21°C in 1 or 2 l of medium containing 0.5% Difco yeast extract (wt/vol) and 2% glucose (wt/vol) in a 2.8-l Fernbach flask on a reciprocating platform [14].

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Table 1. Carotene levels for pink, pumpkin, yellow, and orange haploid strains of *Ustilago violacea*

Strain	Phenotype	Carotene mg/cell × 10^{12}								
		Phytoene	ζ	Neuro-sporene	Lycopene	β-zea	γ	β	Colored carotene	Total carotene
1.C421	Pink	13.5	—	—	2.7	—	—	17.9	20.6	34.1
2.C425	Pink	12.4	—	—	—	1.4	1.9	39.0	42.3	54.7
2.C424	Pink	54.7	—	—	—	0.3	0.5	21.1	21.9	76.6
AB278a-1	Pink	10.2	0.3	—	—	21.2	—	$<10^{-14}$	21.5	31.7
2.C415[a]	Pumpkin	40.1	2.6	1.5	1.3	—	—	13.7	19.1	59.2
1.C2y[b]	Yellow	52.1	1.6	1.3	—	10.6	—	41.1	54.6	106.7
1.C503[c]	Yellow	8.8	—	—	0.7	0.8	—	1.4	2.9	11.7
2.D37291S[a]	Orange	43.0	—	—	5.7	—	4.5	25.8	36.0	79.0
2.D3729S[b]	Orange	57.6	1.4	1.8	11.7	—	—	0.4	13.5	71.1

[a] Mean of two replicates.
[b] Mean of three replicates.
[c] This strain turned yellow after two weeks of growth.

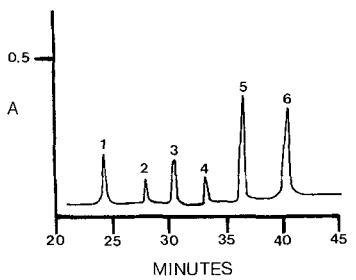

Fig. 1. High-performance liquid chromatography separation of mixed carotene extracts from strains 2.C415, 1.C2y, and 2.D37291S of *Ustilago violacea*, using a C-18 column with a 2-propanol–acetonitrile–H_2O gradient: (1) lycopene, (2) neurosporene, (3) γ-carotene (4) ζ-carotene, (5) β-carotene, (6) cis-β-zeacarotene and phytoene.

In the extraction of carotenes, approximately 30 g (fresh weight) sporidia were dehydrated with acetone and mixed with 30 g of glass beads (16-220, Virtis Co.), 60 ml acetone, 1% butylated hydroxytoluene (2,6-di-tert-butyl-*p*-cresol), and 0.3 g $CaCO_3$, and homogenized for 15 min at 0°C with a Bead Beater (Biospec Products) cell homogenizer equipped with an ice-water cooling jacket [16]. The cell debris was collected by centrifugation at 12,000 *g* for 10 min and exhaustively extracted with acetone until the solvent was colorless. The pooled acetone extracts were evaporated under reduced pressure at 30°C. The residue was then dissolved in approximately 5 ml of petroleum ether (30–60 C bp) and saponified with 30% (wt/vol) ethanolic KOH for 3 h at room temperature. After saponification, 30 ml of distilled H_2O were added prior to the extraction with petroleum ether. The petroleum ether epiphase containing the carotenes was exhaustively extracted with distilled water to remove all traces of KOH, dried over anhydrous Na_2SO_4, and evaporated with a stream of nitrogen.

Carotene extracts were chromatographed on a column (2 × 30 cm) of neutral aluminum oxide (Alumina, Woelm Activity Grade I or III) [16, 21], eluting with petroleum ether (30–60 C bp), and then 1% diethyl ether in petroleum ether. Carotene extracts were also fractionated by high-performance liquid chromatography (HPLC). Extracts were dissolved in a mixture of 30% 2-propanol, 67% acetonitrile, and 7% H_2O, precipitates removed by centrifugation, and a 48-μl sample injected through a rheodyne 7010 injector equipped with a 48-μl loop onto an Altex C-18 column. The carotenoids were eluted with a sigmoidal gradient of 30% 2-propanol and 70% (90% aqueous acetonitrile) to 55% 2-propanol 45% (90% aqueous acetonitrile). The gradient was made with an Isco Model 384 DialaGrad gradient programmer connected to two pumps (Isco High Pressure). At the flow rate of 40 ml/h the operating pressure was initially 63 atm as measured with an Isco model 1590 pressure monitor. The eluant passed through a Perkin Elmer spectrophotometer (model LC-55) equipped with a high-pressure micro cuvette, monitoring either at 425 nm or 283 nm. Elution profiles and retention times were recorded with a Hewlett Packard (HP 3390A) integrator. Fractions were collected, evaporated to dryness under a stream of nitrogen, and scanned for their visible or ultraviolet absorption spectra in an Aminco DW-2 recording spectrophotometer. Carotenes were identified by cochromatography with authentic standards and from their spectra. Carotene concentration was determined quantitatively from their absorption at λ max and the specific extinction coefficient [6].

Results

Carotene extracts from *U. violacea* were inadequately separated into their components by chromatography on Alumina columns. While rechromatography on a second Alumina column increased resolution, purification was incomplete as judged by the spectra of the collected fractions. Satisfactory separation of carotenes from *U. violacea* was achieved by HPLC with a C-18 reverse-phase column eluted with a 2-propanol and aqueous acetonitrile gradient. The elution order of the carotenes was lycopene, neurosporene, γ-carotene, ζ-carotene, β-carotene, cis-β-zeacarotene, phytoene. The separation of carotenes in *U. violacea* extracts was complete (Fig. 1).

The yellow, orange, pumpkin, and pink strains of *U. violacea* accumulated the largest amounts of

Table 2. Carotene levels for ultraviolet-induced and wild, haploid white strains of *Ustilago violacea*

Strain	Carotene concentration mg/cell × 10^{12}						
	Phytoene	Lycopene	cis-β-zea	γ	β	Colored carotenes	Total
Ultraviolet-induced							
2.11.19.76–15.3	2.3	—	—	—	—	—	2.4
1.6.6.7.73–11.5	—	—	—	—	—	—	—
2.7.12.73–15.1	—	—	—	—	—	—	—
2.7.12.73–15.10[a]	—	—	—	—	—	—	—
1.6.22.73–16.1	3.9	—	—	—	—	—	3.9
1.C2w[a]	—	—	—	—	—	—	—
Wild							
1.C427	—	—	—	—	—	—	—
2.C420	7.1	—	—	—	—	—	7.1
2.C449[a]	12.0	—	—	—	—	—	12.0
1.C419	30.2	—	—	—	—	—	30.2
1.C433	—	—	—	—	0.3	0.3	0.3
1.C431	—	—	—	—	$<10^{-14}$	$<10^{-14}$	$<10^{-14}$
1.C430	$<10^{-14}$	—	—	—	3.2	3.2	3.2
1.C418	6.9	—	—	—	0.9	0.9	7.8
1.C504[a]	2.9	—	0.9	$<10^{-14}$	0.8	1.8	4.7
1.C435	16.9	1.4	—	—	2.4	3.9	20.7

[a] Mean of two replicates.

Table 3. Carotene levels for disomic and diploid strains of *Ustilago violacea*

Strain	Genotype	Phytoene	cis-β-zea	β	Colored carotenes	Total carotenes
1.C695	*w/w*	—	—	0.3	0.3	0.3
2.C684-1	*w/y*	22.8	1.0	31.5	35.2	55.3
6.17.75–11.6/p	*w/p*	40.8	—	38.8	38.8	79.6
9.8/1.C2	*w/y*	27.4	7.5	2.3	9.8	37.2
11.4/2.D3729S[a]	*w/o*	22.4	—	7.7	7.7	30.1
JK2[a]	*y/o*	58.7	8.3	3.7	12.0	17.9

[a] Mean of two replicates.

carotenes (Table 1). The yellow strain 1.C2y accumulated phytoene, ζ-carotene, neurosporene, cis-β-zeacarotene, and β-carotene, with phytoene, β-carotene, and cis-β-zeacarotene present in highest concentration (Table 1). The total carotene yield for 1.C2y was 107 × 10^{-12} mg/cell, which is considerably higher than for any other strain tested (Tables 1–3). The yellow strain 1.C503, originally classified as a white strain, became yellow after two weeks of culture. After seven days of culture this strain accumulated phytoene, lycopene, cis-β-zeacarotene, and β-carotene in levels typical of the other colored carotene-containing white strains, which was ten times less carotene than in yellow strain 1.C2y (Tables 1–3). Strain 1.C503 is the only strain in which lycopene and cis-β-zeacarotene were detected together (Tables 1–3).

Orange strains 2.D37291S and 2.D3729S were similar in that they both accumulated phytoene, lycopene, γ-carotene, and β-carotene (Table 1). In strain 2.D37291S, phytoene was present in the greatest quantity (43 mg × 10^{-12} mg/cell), with β-carotene at the level of 25 × 10^{-12} mg/cell (Table 1). The lycopene and γ-carotene concentrations were approximately equal (Table 1). The total carotene yields for the two orange strains were approximately the same, but strain 2.D37291S accumulated almost three times the amount of colored carotene as strain 2.D3729S (Table 1). The strains were also typical of the other lycopene-containing strains in lacking cis-β-zeacarotene.

Pumpkin strain 2.C415 accumulated phytoene, ζ-carotene, neurosporene, lycopene, and β-carotene (Table 1). Phytoene was present in the highest concentration (40.1 × 10^{-12} mg/cell), and β-carotene was the colored carotene present in highest concentration (Table 1). The total carotene yield for pumpkin strain 2.C415 was 59.2 × 10^{-12} mg/cell (Table 1).

In the pink strains, β-carotene and phytoene

were found in the highest concentration, ζ-carotene, lycopene, and cis-β-zeacarotene in considerably lower concentration. The amount of colored carotene in these pink strains was on the order of 20 $\times 10^{-11}$ mg/cell (Table 1). Three of the seven tested pink strains, 1.C415, 1.C429, and 2.C428, contained no detectable carotenes.

Wild white and ultraviolet-induced strains generally contained few, if any, carotenes. Of eight ultraviolet-induced white strains, six lacked carotenes and two contained phytoene at the level of 2–4 $\times 10^{-12}$ mg/cell (Table 2). The wild white strains showed considerably more variation in carotene content than the white strains. Of ten strains, six accumulated trace levels of β-carotene; strain 1.C504 accumulated cis-β-zeacarotenes and γ-carotene as well, and strain 1.C435 was the only white strain with lycopene (Table 2). With the exception of three of the wild white strains (1.C427, 1.C433, and 1.C431) phytoene was accumulated on the order of 10^{-12}–10^{-11} mg/cell (Table 2). In strain 1.C433 and 1.C431, trace amounts of β-carotene were accumulated but no phytoene was detected. The total yield of colored carotenes for the wild white strains ranged from 0 to approximately 4 $\times 10^{-12}$ mg/cell (Table 2). The total yield of carotenes for all of the white strains ranged from 0–30.2 $\times 10^{-12}$ mg/cell. Strain 1.C419 accumulated the highest amount of carotenes containing phytoene only (Table 2).

The total yield of carotene for the diploid and disomic strains was within the range for the haploid colored strains except the *w/w* disomic strain and in the *y/o* diploid strain JK2 (Table 3). Strain JK2 accumulates carotene at the level of some of the white haploid strains. The *w/w* disomic, as expected, accumulated the least amount of carotene (Table 3).

Discussion

High-performance liquid chromatography (HPLC) (10,000 theoretical plates) combines the speed and higher resolution power of thin-layer chromatography (100 theoretical plates) and is ideally suited to separate carotenes. Only a few reports on the HPLC of carotenoids have been published, and they are concerned primarily with the separation of the oxygenated carotenoids [1, 11]. In these cases, the carotenes are either partially separated or eluted as a complete mixture. β-Carotene and lycopene as well as partial β-carotene/α-carotene separations have been reported using reversed phase columns with HPLC [1, 35]. These reports do not provide methods to separate a mixture of unpolar carotenes ranging from phytoene to β-carotene. High-performance liquid chromatography on the C-18 reverse phase column, eluted with the 2-propanol–acetonitrile–H_2O gradient, separated the carotenes efficiently and reproducibly. The HPLC method separates and detects carotenes at a level as low as 1–10 μg, raising the level of detection approximately 100-fold. Previous analyses of carotenes in fungal strains usually detected only the main carotene. Genetic modeling of the biosynthetic pathway of carotenogenesis was, therefore, based on limited information.

Yellow strain 1.C2y accumulated primarily β-carotene, phytoene, and cis-β-zeacarotene. White strain 1.C435, which turns yellow with time, is similar to 1.C2y except it also contains traces of lycopene. This was the only strain containing lycopene and cis-β-zeacarotene. Wild yellow strains of *Phycomyces blakesleeanus*, heterokaryotic for mating type alleles, accumulate a mixture of phytofluene, ζ-carotene, neurosporene, lycopene, γ-carotene, and sufficient levels of β-carotene to account for their phenotype [18]. The yellow phenotype of *Phycomyces* differs from that of *U. violacea* by the lack of cis-β-zeacarotene.

Garber et al. [14] reported that strain 1.C2y accumulated only β-carotene. Thin-layer chromatography may not have been sufficiently sensitive to detect cis-β-zeacarotene, which accumulated at about 25% the level of β-carotene.

Orange strain 2.D3729S was reported to accumulate only γ-carotene [14]. In this study, two separately maintained orange stocks, 2.D3729S and 2.D37291S, gave phytoene, ζ-carotene, neurosporene, lycopene, and β-carotene, and phytoene, lycopene, γ-carotene, and β-carotene, respectively. The spectrum of the nonfractionated carotene extract for both strains prior to HPLC is similar to that of γ-carotene.

Pumpkin strain 2.C417, which accumulated lycopene [16] was not available for study and was therefore substituted with pumpkin strain, 2.C415, which mapped at the same locus. Pumpkin strain 2.C415 accumulated high levels of phytoene and β-carotene but had ten times less lycopene than β-carotene. The yellow-orange phenotype of pumpkin strains may result from the presence of β-carotene and lycopene.

The wild pink strains of *U. violacea* accumulated either no carotene or different levels of two or more carotenes: phytoene, ζ-carotene, lycopene, β-

zeacarotene, γ-carotene, and β-carotene. These pink strains of *U. violacea* were unlike pink or red strains of *N. crassa* and *P. blakesleeanus*, which either accumulated a mixture of phytoene, phytofluene, neurosporene, lycopene, γ-carotene, and β-carotene or primarily lycopene [30]. Typically, in pink strains of *U. violacea*, which did accumulate carotene, the strains contained either phytoene, lycopene, and β-carotene, or phytoene, cis-β-zeacarotene, and β-carotene as the major constituents. Pink strain (AB278a-1) accumulated much more cis-β-zeacarotene than β-carotene, which was not observed in other carotene-accumulating strains.

The ultraviolet-induced white strains of *U. violacea* accumulated either no carotene or only phytoene. About 40% of the wild white strains also fell into these two categories. The remaining strains accumulated low levels of some of the colored carotenes. Garber [12] reported at least two and possibly three complementation regions in the white, *w*, locus of *U. violacea*. Loci for the *w* strains of *U. violacea* with no carotene accumulation map in the complementation region *w*A, proximal to the centromere. The wild *w* strains with no carotene accumulation also map in the *w*A complementation region. Two white strains, 1.C433 and 1.C431, accumulating trace levels of β-carotene and lacking phytoene, are also mapped in the *w*A complementation region [13].

The ultraviolet-induced wild white strains accumulating only phytoene, or additionally one or more of lycopene, cis-β-zeacarotene, γ-carotene, and β-carotene, were located in the white locus on a distal cistron *w*B [12, 13]. Trace levels of the colored carotenes in these strains may result from leaky mutations. The ultraviolet-induced *w* strains in this category accumulated only phytoene and were not leaky.

In *Neurospora crassa*, three albino, *al*, loci have been detected. The closely linked *al*-1, *al*-2, loci separated by the *arg*-5 locus [25, 26, 27] and the other *al*-3 in a different chromosome [32]. The *al*-2 mutants accumulate only phytoene [17], suggesting that the biochemical block in the synthetic pathway involved phytoene dehydrogenase. The alleles in the *al*-1 locus do not complement, suggesting only one enzyme [31]. The *al*-2 mutants lack phytoene and the colored carotenes, indicating a block in the pathway prior to phytoene synthesis [22]. The *al*-3 mutants also lack carotenes, but they map at a different locus than *al*-2 mutants [22].

Two albino loci, *car*A and *car*B, have been detected in *P. blakesleeanus* [30]. *Car*B mutants accumulate only phytoene, and the block is presumably at phytoene dehydrogenase; *car*A mutants do not contain phytoene, indicating a block in the pathway prior to phytoene [30]. It has been hypothesized that the *car*A locus codes for a substrate-transfer protein that transfers the substrates from one enzyme to the next in the enzyme aggregate [30].

The white locus in *U. violacea* is as complex as either the *N. crassa* or *P. blakesleeanus* albino loci [5, 19, 20]. Since the strains of *U. violacea* that lack phytoene map in the first cistron, *w*A of the *w* locus, this cistron may code for an enzyme responsible for the synthesis of phytoene from a precursor [23, 24]. The distal cistron *w*B may code for the phytoene dehydrogenase. In either case, the presence of trace amounts of other carotenes in the few wild strains may reflect leaky mutations.

The diploid and disomic strains of *U. violacea* generally followed the carotene-accumulation characteristics of the parental strains with respect to phytoene, cis-β-zeacarotene, and β-carotene since these were the only carotenes found in the diploid and disomic strains. All of the diploid and disomic strains involving the yellow strain 1.C2y accumulated cis-β-zeacarotene and β-carotene. The *o*/*w* and *p*/*w* diploid strains yielded β-carotene but not cis-β-zeacarotene. The diploid and disomic strains seem to be particularly efficient in metabolizing the intermediates because no other carotenes were found.

Heterokaryons of *P. blakesleeanus* from red and white mutant strains, accumulating either lycopene or phytoene, displayed a range of colors from white to orange. The relative amounts of lycopene, γ-carotene, and β-carotene were related to the proportion of red and white mutant nuclei in the particular heterokaryon [10]. Heterokaryosis with numerous nuclei is clearly very different than diploidy and disomy in *U. violacea*.

Genetically based models for carotene biosynthesis suggest that the enzymes involved are transcribed as one message yielding an enzyme aggregate [30, 34], capable of performing the necessary dehydrogenations and cyclizations. A genetically based model for the enzymes involved in the Porter-Lincoln pathway of carotene biosynthesis in *U. violacea* has been proposed by Garber et al. [14]. In this model with *w* locus coded for dehydrogenase enzymes that converted phytoene to lycopene. The *o* and *p* loci represented subunits of the first cyclase responsible for conversion of lycopene to γ-carotene and γ-carotene to β-carotene. The *y* mutations were proposed to lead to an association of the

cyclase subunits and the dehydrogenases, facilitating the production of β-carotene as the end product [14]. A revised model for carotene synthesis in *U. violacea* has to accommodate the new biochemical information, as well as the genetic regulation of cyclization substrate. In *U. violacea* either neurosporene or lycopene can be cyclization substrate. The model also has to accommodate the observation that phytofluene was not detected in *U. violacea* while in at least some other organisms all of the other intermediates have been detected.

The model for carotene synthesis in *U. violacea*, which accommodates the data presented in this study, as well as that previously presented [14, 16], uses the scheme outlined by Porter and Lincoln [29] and Porter and Anderson [28]. The proximal centromere-linked white locus (*w*A) is responsible for phytoene production; the distal white locus (*w*B) is responsible for the production of phytoene dehydrogenase (DH_1). DH_1 is also responsible for the conversion of phytofluene to ζ-carotene to neurosporene. A second dehydrogenase, DH_2, responsible for the conversion of neurosporene to lycopene, is coded by the *y* locus. The yellow strain 1.C2y has either a mutant or no DH_2 enzyme, and the cyclase uses neurosporene as substrate, converting it to cis-β-zeacarotene. The orange and pumpkin strains use lycopene as a cyclization substrate, converting it directly to γ-carotene. This model stipulates that the preferred pathway for the cyclase is through lycopene. Therefore, when the second dehydrogenase, DH_2, is functional, lycopene rather than neurosporene will be cyclized. This model also assumes that the cyclase can convert γ-carotene to β-carotene. The orange and pumpkin loci have not been separated by recombination, but do complement [14]. We propose intragenic complementation for the *o* and *p* mutations, that is, *o* and *p* may be a complex locus. Further, orange strain 2.D3729S has an almost totally nonfunctional cyclase, resulting in lycopene as the primary accumulating carotene. In the orange strain 2.D37191S, the mutation is leaky, allowing γ-carotene and β-carotene accumulation as well.

If the *y* strain has a mutant DH_2 that cannot convert neurosporene to lycopene, in keeping with the proposed model, then there must be another dehydrogenase, DH_3, that specifically converts cis-β-zeacarotene to γ-carotene. Evidence supporting DH_3 was found in pink strain AB278a-1, which accumulated cis-β-zeacarotene as a terminal product. According to the proposed model, this strain should have the following enzyme content: functional DH_1, mutant DH_2 and DH_3, and functional cyclase. The need for two mutations may account for its infrequent occurrence.

The absence of phytofluene from any carotene extract in *U. violacea* could be explained either by the chemical nature of phytofluene dehydrogenation or the nature of the enzyme system. The conversion of phytofluene to ζ-carotene may be an energetically flavored reaction resulting in the immediate dehydrogenation of phytofluene. If the initial dehydrogenases were in aggregate form, phytofluene may be tightly bound to the aggregate until dehydrogenated. Either explanation accounts for the lack of phytofluene.

The use of either neurosporene or lycopene as the primary cyclization substrate in carotene biosynthesis has been demonstrated in a number of fungi. *Rhizophyletis rosea* does not produce lycopene; instead neurosporene is the cyclization substrate [4]. Davies et al. [7] also demonstrated that neurosporene is the primary cyclization substrate in *P. blakesleeanus*. The mutant strain of *P. blakesleeanus* that accumulates lycopene only does so during log phase growth; by stationary phase, neurosporene is cyclized and β-carotene is produced via β-zeacarotene [30]. Bramley et al. [2] demonstrated that the mutant C115 *car*42 *mad*H107 of *P. blakesleeanus* utilized both pathways. Whether the cyclization of neurosporene or lycopene is catalyzed by separate specific enzymes was not demonstrated.

Our results demonstrate that both carotene pathways in *U. violacea* are genetically controlled. The proposed carotene biosynthesis model accommodates all of the data and suggests only one cyclase.

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